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Cytokine-induced sleep: Neurons respond to TNF with production of chemokines and increased expression of *Homer1a* in vitro

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ABSTRACT

Interactions of neurons with microglia may play a dominant role in sleep regulation. TNF may exert its somnogeneic effects by promoting attraction of microglia and their processes to the vicinity of dendrites and synapses. We found TNF to stimulate neurons (i) to produce CCL2, CCL7 and CXCL10, chemokines acting on mononuclear phagocytes and (ii) to stimulate the expression of the macrophage colony stimulating factor (*M-CSF/Csf1*), which leads to elongation of microglia processes. TNF may also act on neurons by affecting the expression of genes essential in sleep–wake behavior. The neuronal expression of *Homer1a* mRNA, increases during spontaneous and enforced periods of wakefulness. Mice with a deletion of *Homer1a* show a reduced wakefulness with increased non-rapid eye movement (NREM) sleep during the dark period. Recently the TNF-dependent increase of NREM sleep in the dark period of mice with CD40-induced immune activation was found to be associated with decreased expression of *Homer1a*. In the present study we investigated the effects of TNF and IL-1β on gene expression in cultures of the neuronal cell line HT22 and cortical neurons. TNF slightly increased the expression of *Homer1a* and IL-1β profoundly enhanced the expression of *Early growth response 2* (*Egr2*). The data presented here indicate that the decreased expression of *Homer1a*, which was found in the dark period of mice with CD40-induced increase of NREM sleep is not due to inhibitory effects of TNF and IL-1β on the expression of *Homer1a in neurons*.

1. Introduction

Patients with autoimmune diseases suffer from sickness behavior syndrome (SBS), which is characterized by fatigue, malaise, decreased appetite, weight loss, and reduced social activities (Dantzer et al., 2008). A causal link between tumor necrosis factor (TNF) and SBS is suggested because treatment with the soluble TNF receptor p75 or with antibodies against TNF improved fatigue and depression in patients with rheumatoid arthritis (RA), psoriasis, and Crohn's disease (Farahani et al., 2006; Katz et al., 2009; Lichtenstein et al., 2002; Moreland et al., 2006; Taylor and Feldmann, 2009; Tyring et al., 2006). In recent studies on experimental SBS in mice, the immune activation was triggered by anti-CD40 monoclonal antibodies (mAb), which activate the CD40 receptor in B cells and antigen presenting cells including macrophages and dendritic cells. Mice treated with CD40 mAb show a decrease in wakefulness and an increase in non-rapid eye movement (NREM) sleep during the dark period (Gast et al., 2013; Taraborrelli et al., 2011). Inactivation of the TNF receptor 1 gene or treatment with soluble TNF receptor p75 protects mice from CD40 mediated sleep–wake changes, but not (Nimmerjahn et al., 2005) from immune activation (Gast et al., 2013; Taraborrelli et al., 2011).

Abbreviations: CD40L, CD40 ligand; CD40 mAb, anti-CD40 monoclonal antibody; *Egr2*, early growth response 2; *Fosl2*, Fos-like antigen 2; *Homer1a* KO mice, *Homer1a* gene knockout mice; *Jph3*, junctophilin 3; IL-, interleukin-; *M-CSF/Csf1*, macrophage colony stimulating facto5; *Nptx2*, neuronal pentraxin 2; NREM, non-rapid eye movement sleep; *Ptgs2*, prostaglandin-endoperoxide synthase 2; SBS, sickness behavior syndrome; SD, sleep deprivation; siRNA, short interfering RNAs; SSctx, primary somatosensory cortex; TNF, tumor necrosis factor; TNFR1, tumor necrosis factor receptor 1.

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Reciprocal signaling between neurons and microglia may be essential in remodeling of brain circuits including the formation, modification, and elimination of synaptic structures. This concept is supported by evidence that microglia processes periodically contact dendritic spines and axon terminals in vivo (Davalos et al., 2005; Nimmerjahn et al., 2005; Wake et al., 2009). Contacts between neurons and microglia foster cell–cell communication via membrane receptors and soluble mediators including cytokines. Among the latter TNF, is considered to play an important role in behavior and immune-mediated inflammation. In inflammatory diseases TNF is produced mainly by microglia and macrophages. Examples are experimental autoimmune encephalomyelitis and murine cerebral malaria and (Medana et al., 1997; Renno et al., 1995). As shown by *in situ* hybridization the type of cell producing TNF in rats injected intravenously with lipopolysaccharide (LPS) was identified as microglia, but not as neurons and astrocytes (Buttini et al., 1997). Using *in situ* hybridization and immunohistochemistry microglia and macrophages were characterized as the major sources of TNF in middle cerebral artery occlusion in mice (Gregersen et al., 2000). However, immunohistochemical studies show that the physiological role of TNF in sleep regulation may be mediated by TNF production in neurons (Churchill et al., 2008). TNF binds to TNF receptor TNFR1 and TNFR2, both of which are expressed on neurons (Marchetti et al., 2004). The production of TNF is strongly influenced by circadian rhythms. Levels of *Tnf* mRNA and TNF correlate with sleep propensity, that is, high sleep propensity is associated with high levels of *Tnf* mRNA and TNF. Furthermore increased TNF is seen during sleep deprivation (Imeri and Opp, 2009; Kaushal et al., 2012; Krueger, 2008). Central or systemic injections of TNF or IL-1β increase the duration of NREM sleep and the EEG delta power, the latter being an index of sleep intensity (Krueger, 2008; Opp, 2005). In this regard, it is also noteworthy that TNF and IL-1β cause dysregulation of Clock genes with decreased expression of the period genes *Per1*, *Per2* and *Per3* and the PARbZip transcription factors including *Dbp*, *Tef* and *Hlf* (Cavadini et al., 2007).

The regulation of sleep has been studied intensively using sleep deprivation (SD). SD leads to increased expression of, e.g., immediate early genes/transcription factors, mitochondrial genes, genes involved in energy metabolism, and neurotransmitter transporters and receptors (Cirelli, 2009). Transcriptome profiling in inbred mouse strains showed that genetic background affects susceptibility to SD at the transcriptional level. When taking the genetic background into account, expression of *Homer1a* associates with changes in homeostatic sleep need (Franken et al., 2001; Mackiewicz et al., 2008; Maret et al., 2007) Neurons expressing *Homer1a* also express early growth response 2 (*Egr2*/*Krox20*), *Fos*-like antigen 2 (*Fosl2*), prostaglandin-endoperoxide synthase 2 (*Ptgs2*), junctophilin 3 (*Jph3*), and neuronal pentraxin 2 (*Nptx2*) (Maret et al., 2007). Expression of these transcripts equally increases with sleep need. *Homer1a* mRNA increases during spontaneous and enforced periods of wakefulness (Huber et al., 2007; Maret et al., 2007; Nelson et al., 2004). Mice with a deletion of *Homer1a* (*Homer1a* KO mice) show a reduced wakefulness with increased NREM sleep during the dark period (Naidoo et al., 2014, 2012). Previous studies of mice with CD40-induced sleep showed that *Homer1a* expression decreases during the second half of the dark period when mice show increased NREM sleep (Gast et al., 2013). In the cortex of CD40 mAb-treated mice the decline of *Homer1a* in the dark period was associated with a significant depression of *Egr2*, *Nptx2*, and *Fosl2*.

The effects of cytokines on sleep–wake behavior may involve communications of neurons with microglia. Cytokines may regulate intercellular interactions by promoting neurons to produce chemokines, which attract microglia to neuronal dendrites and synapses. In this study, we investigated whether TNF may lead to the expression of chemokines by neurons. The data presented here show that TNF activates neuronal production of CCL2, CCL7 and CXCL10 and of *M-CSF*/*CSF-1*. These chemokines have been well-described to attract mononuclear phagocytes and to lead to the extension of microglia processes. By influencing synaptic strength and by secreting mediators such as glutamate and prostaglandins, TNF-stimulated microglia cells may play a pivotal role in the regulation of sleep. Our study on effects of cytokines on neurons also addresses the question as to weather downregulation of *Homer1a* expression in CD40-activated mice is due to cytokine-mediated repression of *Homer1a*. Such effects would induce transitions between wakefulness and sleep. We found that HT22 cells and cortical neurons respond to TNF and IL-1β with increased *Egr2* and *Ptgs2* expression, but not with a downregulation of *Homer1a*. Thus in CD40 mAb-treated mice, the decrease of *Homer1a* expression in the dark period is not due to direct inhibitory effects of TNF and IL-1β on *Homer1a* transcription.

2. Methods

2.1. Primary cortical neurons, HT22 cells and cytokine treatment

Neurons were isolated from the cerebral cortex of C57Bl/6 J mice gestational stage E14–E18 as previously described (Ogunshola et al., 2002). Dissected cortices were dissociated in Hank's buffered salt solution containing trypsin or papain and DNase I for 5 min at 37 °C. Neurons were seeded on poly-L-lysine coated petri dishes (3×10^6 cells per 100 mm dish) in Neurobasal medium containing B27 supplement (1×), albumax (0.25 g/ml), 1% sodium pyruvate, 100 U/ml penicillin–streptomycin and 1 mmol L-glutamine (GIBCO, Invitrogen, AG, Switzerland). The cultures (purity 98%), were maintained for 17 days at normal atmosphere (21% O_2) in a humidified incubator at 37 °C. For analysis of cytokine expression cells were treated on day 14–17 with TNF (Peprotech, London UK) or PBS control for 4–8 h. Thereafter cells and supernatants were harvested.

For expression of *Homer1a*, *Egr2*, *Ptgs2* and *Fosl2* primary cortical neurons were plated either on 35 mm dishes (at 1.5×10^6 cells; density = 1560 cells/mm^2) pre-coated with 0.1 mg/ml poly-L-lysine. Cultures were maintained in a humidified CO_2 incubator (5% CO_2, 37 °C) and half of the medium was changed once a week. After treatment of cortical neurons with cytokines or PBS control during 4 h, total RNA from cell cultures was extracted.

The mouse hippocampal neuronal cell line HT22 was obtained from David Schubert at the Salk Institute (La Jolla, CA). HT22 cells were plated in 12-well tissue cultures plates (100,000 cells per well) in DMEM with 10% FCS. Two days after plating, cultures were serum deprived for 1 h. Thereafter, HT22 cells were treated with TNF or IL-1β for 4 h and RNA extracted.

2.2. RNA extraction and quantification

RNA was extracted using RNA easy mini kit (Qiagen). All RNA samples were DNase-treated and quantified on a NanoDrop ND-1000 spectrophotometer. To quantify the RNA expression level, 1 μg of RNA was reverse-transcribed in 20 μl using random hexamers and Superscript II reverse transcriptase (Invitrogen) according to standard procedures. The cDNA was diluted 10 times and 2 μl were amplified in a 10 μl TaqMan reaction on ABI PRISM HT 7900 detection system in technical triplicate. Cycler conditions were 50 °C 2 min, 95 °C 10 min and 45 cycles at 95 °C 15 s and 60 °C 1 min. Forward primer, reverse primer, and probe sequences are given in Table 1. The gene expression level was normalized to three reference genes (*Rsp9*, *TBP*, and *EEF1a1*) using Qbase software (Hellemans et al., 2007). The fold changes indicative of the relative

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Table 1
Primer sequences for RT-qPCR.

Gene	Forward primer 5′→3′	Reverse primer 5′→3′	Probe 5′→3′
EEF1a1	CCTGGCAAGCCCATGTGT	TCATGTCACGAACAGCAAAGC	TGAGAGCTTCTCTGACTACCCTCCACTTGGT
Homer1a	GCATTGCCATTTCCACATAGG	ATGAACTTCCATATTTATCCACCTTACTT	ACACATTCAATTCAGCAATCATGA
RPS9	GACCAGGAGCTAAAGTTGATTGGA	TCTTGGCCAGGGTAAACTTGA	AAACCTCACGTTTGTTCCGGAGTCCATACT
TBP	TTGACCTAAAGACCATTGCACTTC	TTCTCATGATGACTGCAGCAAA	TGCAAGAAATGCTGAATATAATCCCAAGCG
Egr2	AGGCCCCTTTGACCAGATG	CTTCTCTCCAGTCATGTCAATGTTG	CGGAGTGGCGGGAGATGGCAT
Fosl2	AGTGATCAAGACCATCGGTACCA	CTCCGGATTCGACGCTTCT	CCGCAGAAGGAGAGATGAGCAGCTGT
Ptgs2	AGCGAGGACCTGGGTTCAC	TGTCCAGAGTTTCACCATAAATGTG	AGGACTGGGCCATGGAGTGGACTTAAA

gene expression are based on the mean of 3 biological replicates in relation to control samples. The statistical analysis was performed with a one-way Anova. To determine the changes in gene expression form the control group a Bonferroni post hoc test was performed.

2.3. Gene expression arrays

Cytokine and chemokine gene expression was tested using a cytokine gene array (Gene Expression System StellARray; Bar Habor Biotechnology) according to the manufactures instructions. The results were analyzed with the "Global Pattern Recognition" (GPR) Tool from Bar Habor Biotechnology. All array analyses were performed in triplicates.

2.4. Cytokine analysis

Cytokines were measured in supernatants of cell cultures by Luminex technology (BioRad) following the manufacturer instructions.

3. Results

3.1. Production of chemokines by TNF treated neurons

To assess whether in cortical neurons TNF leads to the production of a set of cytokines and chemokines, which act on mononuclear phagocytes rather then on lymphocytes, we studied the expression of cytokines using cytokine gene arrays. Our data show that TNF enhanced the expression of only a very limited number of cytokine genes. Out of 96 genes represented on the microarray only 7 transcripts were found to be upregulated more than 2-fold. TNF induced the expression of the chemokines *Ccl2*, *Ccl5*, *Ccl7*,*Cxcl1*, *Cxcl5* and *Cxcl10* (Table 2).

The only non-chemokine gene activated in cortical neurons was the *macrophage colony stimulating factor* (*M-CSF/Csf1*). We validated the gene array data obtained by quantifying the chemokine levels in the culture supernatants. In agreement with the gene chip data, supernatants of TNF-treated cortical neurons showed increased concentrations of CCL2 (MCP1), CCL7 (MCP3) and CXCL10 (IP-10) (Table 3). CXCL1 was also induced by TNF, although the effect did not reach statistical significance. In contrast CCL5 (RANTES), and CXCL5 (LIX) were only increased at the mRNA level. *M-CSF* was detected in cortical neuron supernatants, but its production was not regulated by TNF.

Using gene array the cortical neurons treated with TNF were not found to show an increased expression of (i) proinflammatory cytokines (*IL-1α/β*, *IL-6*, *GM-CSF/Csf1*, *G-CSF*), (ii) cytokines involved in T cell development and activation such as *IL-12*, *IL-17*, *IL-23*, *TGFβ*, *IFNγ*, *IL-4* and *IL-5*, and of (iii) cytokines that promote B cell lineage development, differentiation and activation (*IL-6*, *BAFF and APRIL*).

3.2. Expression of Homer1a, Egr2, Ptgs2 and Fosl2 in HT22 cells and cortical neurons

To determine whether TNF and IL-1β modulates the expression of *Homer1a*, *Egr2*, *Ptgs2* and *Fosl2*, we treated the neuronal cell line HT22 with TNF for 4 h. As shown in Fig. 1 TNF strikingly upregulated *Ptgs2* and increased *Egr2* in a dose dependent manner, both genes being most affected with a TNF concentrations of 10 ng/ml. HT22 cells responded to the treatment with TNF with a slight increase of *Homer1a*, and did not react to the cytokine with a dose dependent increase of *Fosl2* (above 2.0-fold).

Besides TNF-α, also IL-1β is produced by CD40 mAb-treated macrophages and dendritic cells and has been implicated in increased NREM sleep and dysregulation of clock gene expression (Cavadini et al., 2007; Taraborrelli et al., 2011). Therefore, we examined whether the effect of TNF described here is a unique property of TNF or is shared by IL-1β. HT22 treatment with IL-1β only moderately increased *Fosl2*, but had no effect on *Homer1a*, *Ptgs2* and *Egr2* (Fig. 2).

However when using primary cortical neurons and treating them with IL-1β the expression of *Egr2* was enhanced (Fig. 3). While the expression of *Homer1a* and *Fosl2* remained unchanged irrespective of the dose of IL-1β added to the neuronal cultures,

Table 2
TNF up-regulated cytokine genes in cortical neurons.

Gene	Gene symbol	Cortical neurons	
		Fold change	*P*-value
Chemokine (c-x-c motif) ligand 10	Cxcl10	184	2.77^{-09}
Chemokine (c-c motif) ligand 2	Ccl2	108.4	7.96^{-07}
Chemokine (c-c motif) ligand 5	Ccl5	35.56	2.06^{-06}
Chemokine (c-c motif) ligand 7	Ccl7	19	4.34^{-08}
Chemokine (c-x-c motif) ligand 1	Cxcl1	11.72	4.48^{-07}
Chemokine (c-x-c motif) ligand 5	Cxcl5	5.78	6.33^{-05}
Macrophage colony stimulating factor	Csf1	2.77	0.0002

Data show the cytokine genes that meet the significance cutoff set at 2.0-fold change and *p*-value <0.001

Table 3
Productions of cytokines by TNF treated neurons.

Cytokine	Cortical neurons		Stimulation index (SI)
	Control	TNF	
CXCL10	100.6 ± 43.5	2237 ± 382*	22.3
CCL2	49 ± 19.5	332 ± 96*	6.8
CXCL1	9.2 ± 6.7	48.7 ± 35.7	5.2
CCL7	16.7 ± 0.2	48.5 ± 8.3*	2.9
M-CSF	184.6 ± 6.6	200.6 ± 65.4	1
CCL5	<10	<10	–
CXCL5	<10	<10	–

Cortical neurons were treated for 6 h with TNF. Thereafter supernatants were collected and tested for the cytokines indicated. Data (pg/ml) are given as means ± SD of triplicates.

* $p < 0.01$.

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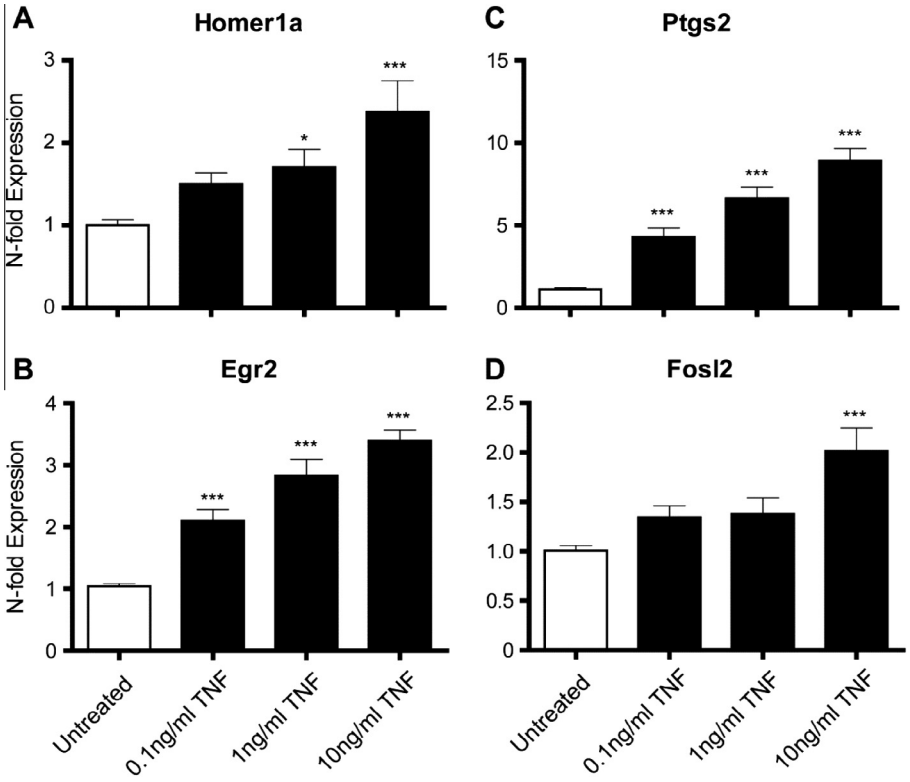

Fig. 1. Treatment of HT22 cells with TNF induces the expression of *Homer1a*, *Egr2*, *Ptgs2* and *Fosl2*. The hippocampal neuronal cell line HT22 is treated with TNF, (0.1 ng/ml; 1 ng/ml and 10 ng/ml) for 4 h (black bars). Compared to PBS control (white bars), treatment with TNF (10 ng/μl) increases the expression of *Homer1a* (A) *Egr2* (B) *Ptgs2* (C) and *Fosl2* (D) by a factor of 2.4, 3.3, 8.1 and 2 respectively. Data of RT-qPCR assays of *Homer1a*, *Egr2*, *Ptgs2* and *Fosl2* expression shows the mean S.E.M of triplicates from four independent experiments. One way Anova with Bonferroni post hoc test: *<0.05; **<0.01; ***<0.001.

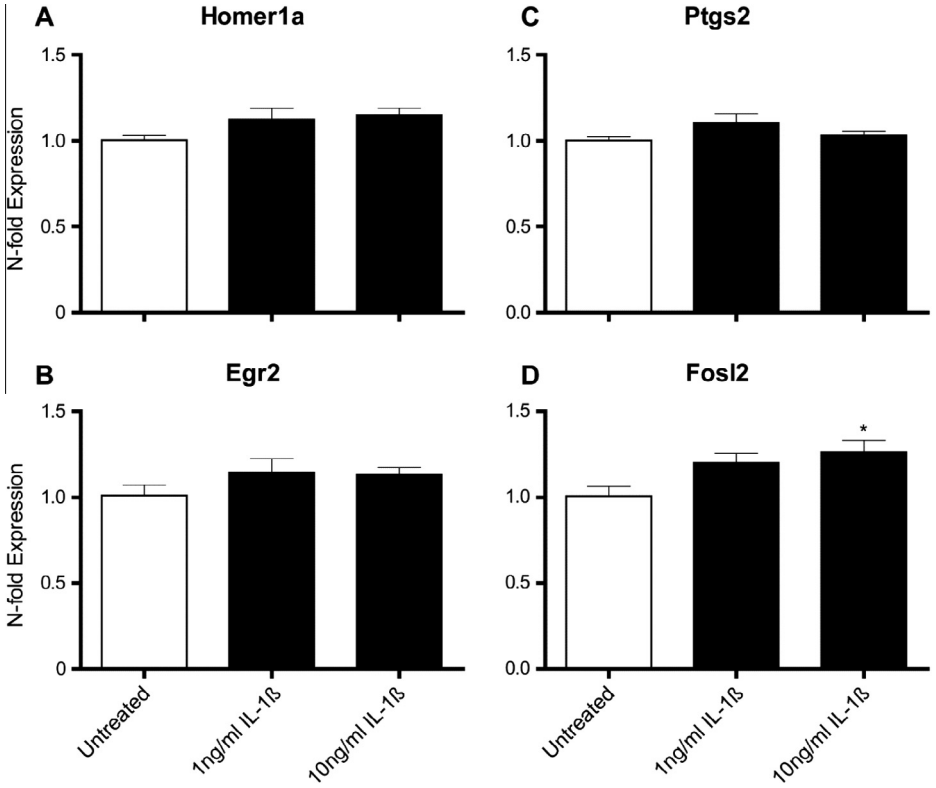

Fig. 2. Treatment of HT22 cells with IL-1β slightly increased the expression of *Fosl2*, but not of *Homer1a*, *Egr2* and *Ptgs2*. HT22 cells are treated with either 1 ng/ml IL-1β or with 10 ng/ml IL-1β for 4 h (black bars). Compared to PBS controls (white bars), none of the four genes tested was increased by IL-1β above 2-fold. Data of RT-qPCR assays of *Homer1a*, *Egr2*, *Ptgs2* and *Fosl2* expression shows the mean S.E.M of quadroplates for two independent experiments. One way Anova with Bonferroni post hoc test: *<0.05.

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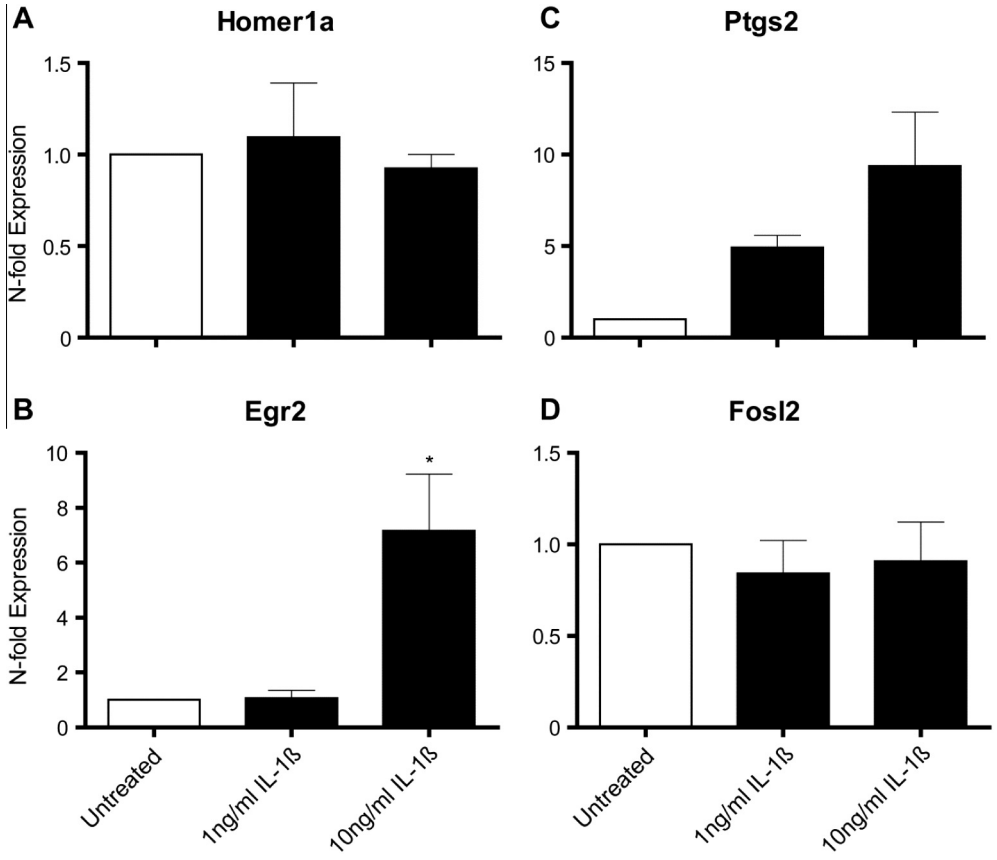

Fig. 3. Treatment of primary cortical neurons with IL-1β enhanced the expression of *Egr2*, but does not affect the expression of *Homer1a* and *Fosl2*. Cortical neurons are treated with either 1 ng/ml IL-1β or with 10 ng/ml IL-1β for 4 h (black bars). Compared to controls (white bars), IL-1β (10 ng/μl) highly upregulated *Egr2*. The stimulatory effects on *Ptgs2* did not reach statistical significance. Data of RT-qPCR assays of *Homer1a*, *Egr2*, *Ptgs2* and *Fosl2* expression shows the mean S.E.M of quadroplates for two independent experiments. One way Anova with Bonferroni post hoc test: *<0.05.

Ptgs2 mRNA was increased, the effect, however, not reaching statistical significance.

4. Discussion

4.1. Cortical neurons, treated with TNF produce M-CSF/CSF-1 and the chemokines CCL2, CCL7 and CXCL10

The question arises as to how the production of somnogenic cytokines is induced in microglia cells in the physiological sleep wake cycles. A central role may be attributed to ATP. Neuronal activity has been found to be associated with synaptic co-release of ATP and neurotransmitters. ATP acts via P2×7 receptors on microglia to induce the release of TNF and IL-1β (Krueger, 2008). The pathway proposed requires direct interactions of neurons with microglia, the main producers of TNF in the brain. Cooperative interactions of the two types of cells may also be essential for microglia-mediated synaptic plasticity including dendritic spine remodeling and elimination (Tremblay and Majewska, 2011; Tremblay et al., 2011). Following visual stimulation microglia sample individual synapses more frequently compared with nonstimulated synapses (Tremblay et al., 2010). Thus microglia may monitor the functional state of synapsis and contribute to plastic changes (Blank and Prinz, 2013). Synaptic strength is suggested to occur during wakefulness, downscaling of synaptic strength to basal level may play a fundamental role in sleep (Tononi and Cirelli, 2006). In the study presented here we determine whether neurons produce factors, which attract microglia cells. The results demonstrate that primary cortical neurons respond to TNF treatment with upregulated expression of the chemokine genes *Ccl2*, *Ccl5*, *Ccl7*, *Cxcl1*, *Cxcl5* and *Cxcl10*. At the protein level only CCL2, CCL7 and CXCL10 were upregulated. Reports on neurons as a possible source of chemokines are sparse, but indicate that they can make a contribution. Measles virus infection of primary hippocampal neurons induced CXCL10 and CCL5 production (Patterson et al., 2003). Scrapie infected neurons mainly upregulated expression of CCL5, but also induced lower levels of other chemokines including CCL2 (Marella and Chabry, 2004). The chemokines identified here to be secreted by cortical neurons – CCL2 and CCL7 – attract cells of the monocyte-macrophage lineage. CCL2 and CCL7 bind to the chemokine receptor CCR2, which is expressed in microglia as well as on neurons (Banisadr et al., 2005; Boddeke et al., 1999). By attracting microglia, the neuronal production of CCL2 and CCL7 may play a pivotal role in neuron-microglia communication. CCL2 has also been shown to induce migration of neuroprogenitor cells and directs neural progenitor cell migration following striatal cell loss (Gordon et al., 2009; Vrotsos and Sugaya, 2009). TNF treatment of cortical neurons increased the production of CXCL10. This chemokine is induced in neurons by infection with dengue virus and inhibits binding of the virus to cell surface heparan sulfate, a co-receptor for the virus (Ip and Liao, 2010). CXCL10 binds to CXCR3 on microglia cells and induces their migration (Rappert et al., 2004). Besides its chemotactic activity on mononuclear phagocytes, CXCL10 leads to recruitment of activated $CXCR3^+$ T cells, but not resting T cells, that do not express the CXCR3 (Muller et al., 2010). Thus in the physiologic rhythmic expression of TNF in the brain, the functional property of neuronal derived CXCL10

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is confined to its activity on microglia cells rather then T cells. In this context it is of note that TNF did not upregulate the expression of the lymphoid chemokines CCL9, CCL21 and CXCL13, which are critical in the generation of adaptive immune responses (Lalor and Segal, 2010). Our cytokine gene array data also show that cortical neurons did not respond to TNF treatment with upregulated expression of proinflammatory cytokines *(IL-1α/β*, *IL-6*, *TNF*, *GM-CSF*, *G-CSF*), or of cytokines involved in regulation of lineage development of naive T cells into Th1, Th2, Th17 and regulatory T cells (*IL-12*, *IL-23*, *TGFβ*, *IFNγ*, *IL-4* and *IL-5*). Moreover primary neurons failed to respond to TNF with expression of cytokines that promote B cell and plasma cell survival and expansion such as *IL-6, B cell activating factor Baff* and *the proliferation-inducing ligand* (*APRIL*). The lack of TNF-induced neuronal signals, which act on T and B cells may provide a safety strategy of the CNS aimed at prevention of immune priming and lymphocyte activation. Besides of chemokines we found neurons to secrete *M-CSF*/*CSF-1*, a growth factor for microglia cells. For neuronal-microglia communication it is interesting that *M-CSF*/*CSF-1* leads to elongation of microglial processes (Smith et al., 2013). Besides of microglia cells the *CSF1* receptor is expressed in a small number of neurons in the hippocampus and cortex (Luo et al., 2013).

4.2. TNF and IL-1β do not downregulate Homer1a

Homer1a belongs to the plasticity-regulated genes that are upregulated as immediate-early genes during hippocampal long-term potentiation and epileptic seizures. Recent data suggest that *Homer1a* plays a pivotal role in the mechanisms, which regulate sleep. *Homer1a* mRNA is increased in the dark phase in the somatosensory cortex of rats (Nelson et al., 2004) and is up-regulated with sleep loss (Nelson et al., 2004), the extent of the effect being dependent on the strain of mice and associates with sleep pressure (Mackiewicz et al., 2008; Maret et al., 2007). Moreover *Homer1a* KO mice fail to sustain long bouts of wakefulness (Naidoo et al., 2014). Our recent data on *Homer1a* expression in untreated mice are in agreement with the aforementioned studies. *Homer1a* increased with sleep need, lowest levels being observed when mice were resting (Gast et al., 2013). CD40 mAb treatment was found (1) to shorten the time of wakefulness during the dark phase, and (2) to lead from Zeitgeber ZT13 to ZT18 to an early decrease of *Homer1a* expression in the frontal cortex. Likewise intraperitoneal injections of TNF at ZT9 decreased wakefulness within hours and lowered the expression of *Homer1a* at ZT18. In the light of the failure of *Homer1a* KO mice to keep wakefulness the decrease of *Homer1a* after CD40mAb or TNF injections may result from TNF or IL-1β mediated inhibition of *Homer1a* expression. Alternatively the decrease of *Homer1a* may be the consequence of lower sleep pressure in the second phase of the dark period. Since investigations on regulation of *Homer1a* expression by proinflammatory cytokines are lacking, we treated cultured neurons with cytokines and assessed the expression of *Homer1a*. Our in vitro data show that neither TNF nor IL-1β suppressed the expression of *Homer1a* mRNA in HT22 neuronal cells and primary cortical neurons respectively. In fact the opposite was true. *Homer1a* mRNA was up-regulated in HT22 cells treated with TNF, an effect not being seen with IL-1β. When taking the genetic background of different mouse strains into account sleep loss-induced transcriptional changes involved only a limited number of genes (Maret et al., 2007). In addition to *Homer1a* also *Egr2*, *Ptgs2 and Fosl2* were identified to consistently follow the altered expression of *Homer1a* during SD. As true for *Homer1a*, we found TNF and IL-1β to increase rather then to decrease the expression of these genes in HT22 and/or primary cortical neurons. In this respect it is of note that TNF has already been reported to enhance the expression of *Egr1* and *Fos-1* in cultures of vascular smooth muscle cells. These genes are like *Egr2* and *Fosl2* other members of the *Egr* and *Fos* family of transcriptional regulators respectively (Goetze et al., 2001). Moreover *Ptgs2*, the gene encoding cyclooxygenase-2 has been well described to be induced by proinflammatory cytokines including TNF and IL-1β (Cao et al., 2011). These data are in line with the results presented here showing TNF to upregulate *Egr2* and *Fosl2*.

5. Conclusion

Our data on the response of neurons to TNF show that cortical neurons exposed to TNF produce chemokines (CCL2, CCL7, and CXCL10) and *M-CSF/CSF-1*. These factors have been described to attract microglia and to lead to extension of microglia processes. Thus, TNF may play a pivotal role in the neuronal-microglia communication, which allows synaptic plasticity and regulate sleep. The increase of NREM sleep induced by cytokines has been shown to be associated with decreased expression of *Homer1a*. This splice form of Homer1 has been shown recently to be required for maintenance of wakefulness. Our data using cultures of HT22 cells and primary cortical neurons show that TNF and IL-1β do not interfere with *Homer1a* expression in neurons in vitro. In the light of the complex molecular pathways involved in sleep regulation, the interpretation of these in vitro data must be done carefully. The in vitro data shown, however, do not support the hypothesis that the altered expression of *Homer1a* in cytokine-induced sleep is due to direct effects of cytokines on expression of *Homer1a* in neurons in vivo.

Author contributions

M.K., M.A.L., D.M., C.M. and O.O.O. designed and performed the experiments. A.M., L.S., M.T. and A.F. contributed to the concept of the study.

Competing interests

The authors have no competing interests to disclose.

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